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Description

Prophylactic Antiviral and Immunomodulatory Therapy for Prevention of Respiratory Syncytial Virus Infection

BACKGROUND

[0001] FIELD OF INVENTION

[0002] This invention pertains to the development of a prophylactic therapy (PAIT) with vector-delivered short-interfering RNA aimed at preventing severe respiratory syncytial virus infection and while enabling immunomodulation for future protection.

[0003] Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection in infant, young children and the elderly with immunocompromise⁽¹⁾, and is also a risk factor for the development of asthma⁽²⁾. Previous RSV infection does not prevent subsequent infections, even in sequential years⁽³⁾. In the United States only, the severe

viral bronchiolitis and pneumonia results in approximately 100,000 hospitalizations and 4500 deaths in infants and young children each year^(4, 5). To date, there are no specific antiviral treatments available. Although many different approaches are being taken to develop prophylactic vaccines, none have been licensed for public health use to prevent diseases associated with RSV infection.

[0004] RSV is the prototype member of the Pneumovirus genus of the Paramyxoviridae family and is an enveloped nonsegmented negative-stranded RNA virus. The RSV genome of approximately 15,200 nucleotides is transcribed into 10 subgenomic mRNAs, which encodes 11 distinct viral proteins in the order: NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2, and L. Three RSV envelope glycoproteins involves the fusion F protein, the attachment glycoprotein G and the small hydrophobic SH protein. An unglycosylated matrix M protein is present as an inner virion protein. And the nucleocapsid is composed of the major nucleocapsid protein N, P phosphoprotein, large L polymerase subunit and M2-1 protein. Two nonstructural proteins NS1 and NS2 are expressed from separate mRNAs encoded by the first and second genes, respectively, that follow the 44-nt

leader region^(1, 6). As their promoter-proximal location, these two mRNAs are the most abundant of the RSV transcripts in a linear start-stop-restart mode⁽¹⁾. Deletion of either NS gene severely attenuates RSV infection *in vivo* and *in vitro*, indicating that NS proteins play an important role in viral replication cycle (7, 8, 9, 10).

SUMMARY OF INVENTION

[0005] Respiratory syncytial virus (RSV) belongs to Paramyxoviridae family of enveloped negative-strand RNA viruses and causes severe lower respiratory tract infection in children younger than 2 years of age. Currently, there are no licensed vaccine available and prophylactic treatments include treatment with antibodies that are moderately effective and expensive. In this invention attenuating RSV infection using DNA vector-based short interference RNAs (siRNA) was examined in A549 cells. A cassette encoding siRNA targeted to a NS1 no-structural gene was tested for its ability to attenuate RSV infection. A549 cells transfected with siRSV NS1 showed a significant reduction of RSV production in a dose-dependent manner, which provides a basis for the development of siRSV NS1 as potential prophylaxis and therapy for RSV infection in human.

BRIEF DESCRIPTION OF DRAWINGS

[0006] For a fuller understanding of the invention, reference should be made to the following detailed description, taken in connection with the accompanying drawings, in which:

[0007] Figure 1. (A) Diagram of the construction of the plasmid vector, pSMWZ-1, capable of expressing a RSV infection suppressor cassette. Abbreviations: N*, Not I; K, Kpn I; A, Apa I; E, EcoR I; SUP-1, Suppressor cassette; (B) Immunoblotting analysis of NS1 protein expression. Transfected A549 cells in six-well culture plates were infected with rgRSV at an MOI of 1. The infected cells were lysed in protein lysis buffer 24h post-infection and cell lysates were electrophoresed on 12% polyacrylamide gels and the blot was hybridized with a RSV polyclonal antibody. Protein loading controls were performed by probing the blot with monoclonal antibody against actin. (C) siRSV NS1 does not decrease EGFP expression in cultured A549 cells. Cells were seeded into six-well plates and incubated at 37°C/5% CO₂ to 95% confluency. Cells were cotransfected with pEGFP and siEGFP or siRSV NS1 and, after 2 days, the cells were harvested and quantified by flow cytometry.

[0008] Figure 2. (A) Effect of siRSVNS1 on RSV infection in A549 cells. A549 cells were seeded into six-well tissue plates

and incubated at 37°C/5% CO₂ to 95% confluency. Then cells were transfected with either 2µg of siRSV NS1, and the transfected cells were infected with engineered RSV carrying the EGFP gene 24h later(MOI=1). The EGFP positive cells were visualized using fluorescence microscopy and counted in 15 random fields; (B) Flow cytometry analysis of rgRSV positive A549 cells. A549 cells were seeded into six-well tissue plates and cultured as above, then cells were transfected with either 2µg or 4µg of siRSV NS1, and the transfected cells were infected with the same amount of engineered RSV carrying the EGFP gene 24h later. The cells were harvested 20h postinfection and the numbers of RSV infected cells were quantified by flow cytometry.

[0009] Figure 3. siRSVNS1 decreases viral titers and viral replication. (A)Measurement of virus titer by modified plaque assay. Five 10-fold serial dilutions were made for each supernatant sample from infected A549 cells and inoculated into duplicate wells of 96-well tissue culture plates containing confluent A549 cell monolayers. After virus attachment for 1 h, the A549 cells were replaced with fresh medium and incubated for 2 d at 37°C in a 5% CO₂ incubator. The green color cells were counted under fluores-

cence microscopy. Data are the averages of two independent experiments. (B) Quantification of NS1 in antigenome by RT-PCR assay. The total RNAs were extracted and then subjected to reverse transcription followed by PCR performance. The amplification condition were 94°C denaturation for 5min followed by 30 cycles of amplification conditions were 94°C for 50s, 64°C for 50s and 72°C for 50s. The conditions for PCR to detect β -actin were as the same as above except the annealing temperature was set at 56°C.

[0010] Figure 4. Cell viability measurement by MTT assay. A549 cells were seeded into six-well tissue plates and incubated at 37°C/5% CO₂ to 95% confluency. Then cells were transfected with different amount of plasmid DNAs, and the transfected cells were added with MTT solution and incubated for 2h at 37°C. The optical density of resulting purple solution was spectrophotometrically measured at 570 nm.

[0011] Figure 5. siRSVNS1 increases expression of IFN-stimulated genes in epithelial cells. Transfected A549 cells in six-well culture plates were infected with rgRSV at an MOI of 1. The infected cells were lysed in protein lysis buffer 24h postinfection and cell lysates were electrophoresed on

12% polyacrylamide gels and the blots were separately incubated with antibodies to IRF1, IRF3, IRF7, STAT1, STAT6 or STAT2 . Protein loading controls were performed by probing the blot with monoclonal antibody against actin (not shown).

DETAILED DESCRIPTION

[0012] An effective approach to prevent severe RSV infection comprises a reduction in virus titer with concomitant increase host's natural antiviral response, i.e., production of type-1 IFNs. Many viruses have evolved ways to interfere with type-1 IFN response including RSV. RSV NS1 and NS2 genes are known to interfere with type-1 IFN response. We examined the potential of an anti-sense RNA approach aimed at blocking NS gene expression might attenuate RSV virus replication in cells, simultaneously augmenting the type-1 IFN response in these cells. In this study, we examined using A549 alveolar type-II epithelial cells the potential of a DNA-based siRNA approach for downregulating NS1 gene expression *in vitro*. MATERIALS AND METHODS *Virus and cell lines* Recombinant rgRSV was kindly supplied by Dr. Mark E. Peeples (11). The EGFP gene flanked by RSV gene-start lies at the boundary between the 44-nucleotide leader region and the NS1 gene. The

recombinant rgRSV virus expressed one additional mRNA, namely EGFP, compared with wild-type RSV. Human lung epithelial tumor cell lines A549 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cell lines were grown in Earle's modified Eagle's medium (EMEM) supplemented with 10% fetal bovine serum (FBS), incubated under 5% CO₂ at 37°C.

[0013] *Plasmid constructs* The pCMV-MCS plasmid (Stratagene) was digested with Not I and the larger fragment was ligated to the synthetic adapter, containing, in order, Not I-Kpn I-Apa I-Xho I-Hind III-EcoR I-Bam HI-Sac II-Sac I-Cla I-Sal I-Bgl II-Not I; The U6 promoter was obtained by PCR amplification, using specific primers with the desired restriction sites from the template pSilencer 1.0-U6 (Ambion), and inserted into the adaptor at the Kpn I and Apa I sites to get a novel plasmid pCMV-U6. Pairs of oligos were synthesized to develop si-RNA constructs. The nucleotide sequence for each siRNA is as follows: siEGFP: 5"GGC GAT GCC ACC TAC GGC AAG CTT CTC GAT TCG AAG CTT GCC GTA GGT GGC ATC GCC CTT TTT G-3' (12); siRSV NS1:5"-GGC AGC AAT TCA TTG AGT ATG CTT CTC GAA ATA AGC ATA CTC AAT GAA TTG CTG CCT TTT TG-3"; siHPV18 E7: 5"-GAA AAC GAT GAA ATA GAT GTT CAA

GAG ACA TCT ATT TCA TCG TTT TCT TTT TT-3"; Each pair of oligos was annealed and then inserted into pCMV-U6 digested with Apa I/Xho I and Xho I/EcoR I respectively. The modified pCMV-U6 plasmid was then redigested with Not I and the smaller fragment was ligated to the 2.9 kb fragment of pAAV-MCS (Stratagene) obtained following its Not I digestion to generate the corresponding siRNA for EGFP, RSV NS1 and HPV18 E7.

[0014] *DNA transfection and virus infection* 0.8×10^6 A549 cells were seeded into the six-well tissue culture plates and incubated at 37°C/5% CO₂ until 95% confluence and transfected with either 2 µg or 4 µg of siRSV NS1 plasmid DNA using Lipofectamine 2000 reagent (Invitrogen life tech.). The cells were then infected with rgRSV at a multiplicity of infection of one (MOI=1) 1 day post-transfection. pAAV-EGFP plasmid (Stratagene) was used for transfection ratio measurement.

[0015] *Flow cytometry* The cells were harvested 1 day post-infection and centrifuged for 5 min at 500 g. The cell pellets were washed with PBS twice and resuspended with 1% paraformaldehyde and kept on ice for 30min. The numbers of rgRSV infected cells were quantified by Flow cytometry.

[0016] *Modified plaque assay* A modified plaque assay was performed to detect virus titer. The supernatants from rgRSV-infected A549 were collected at day 2 post-infection. 10-fold serial dilutions of the supernatants were added to a monolayer of A549 cells for 1 h absorption and the medium in each well of six-well culture plates was then removed and replaced by a fresh cell culture medium (DMEM, 10%FBS, Non-Essential Amino Acids (Gibco BRL)) and the plates were incubated at 37°C/5% CO₂ for 2 days. Afterwards, the green cells were counted.

[0017] *Protein expression analysis by Western blotting* Western blot assay was used to monitor viral protein expression after siRSV NS1 treatment. Transfected A549 cells in six-well culture plates were infected with rgRSV at an MOI of 1. The infected cells were lysed in protein lysis buffer (150mM NaCl, 25mM Tris-HCl [pH8.0], 0.1mM EDTA, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS, 1mM PMSF, aprotinin[1 µg/ml], leupeptin[1 µg/ml] and pepstain[1 µg/ml]) 24h postinfection. The cell lysates were electrophoresed on 12% polyacrylamide gels (BIO-RAD) and the proteins were transferred to Immun-Blot PVDF membranes (BIO-RAD). The blot was hybridized with a RSV polyclonal antibody (AB1128, Chemicon Int Inc.). This antibody rec-

ognizes all the RSV antigens protein. Immunoblot signals were developed by Super Signal Ultra chemiluminescent reagent (Pierce, Rockford, IL.) according to the manufacturer's instructions. Protein loading controls were performed by probing the blot with monoclonal antibody against β actin (Clone ACTN05, LAB VISION Co.).

[0018] In separate experiments, the proteins were isolated at various time points after RSV infection and proteins were blotted using antibodies to various IFN-regulated proteins.

[0019] *Cell viability measurement by MTT assay* The effect of plasmid DNA on the viability of A549 cell was determined using MTT kit (Sigma Chemical Co.). A549 cells were plated in duplicated in six-well culture tissue plates and transfected with either siRSV NS1 or siHPV18 E7 or pSMWZ-1 plasmid DNA, and the growth inhibition were measured after 24 h of exposure. Cell proliferation was evaluated as a fraction of cell growth in plasmid DNA-free control group.

[0020] *Analysis of NS1 gene in antigenome by RT-PCR assay* Total RNA was extracted by RNeasy Mini Kit (QIAGEN) according to the manufacturer's recommendations and then subjected to reverse transcription using a random primer mix and SuperScript II RNase H- reverse transcriptase

(Invitrogen) followed by PCR performance as descriptions previously (13). RSV NS1 primers were 5"–CTG ACG GGA TCC GAA TTC AGG ATG GGC AGC AAT TCA TTG–3" (forward) and 5"–GGC ATT CTC GAG TTA TGG ATT AAG ATC AAA TCC AAG TAA–3" (reverse). The β -actin primers were 5"–CGC GAG AAG ATG ACC CAG–3" (forward) and 5"–ATC ACG ATG CCA GTG GTA C–3" (reverse).

[0021] RESULTS

[0022] *Development of a DNA vector-based siRNA delivery system for gene silencing* In order to develop a plasmid–siRNA system, a plasmid, pSMWZ–1, was engineered that comprised a mouse U6 promoter linked to a siRNA cassette (Fig. 1A). For detection of whether siRSV NS1 decreases rgRSV virus NS1 protein expression in cultured A549 cells, cells were transfected with different dose of siRSV NS1 plasmid DNA or siHPV18 E7 (control) silencing cassettes. 1 day post transfection the cells were infected with rgRSV virus at an MOI of 1. After 1 day, the infected cells were harvested and lysed with protein lysis buffer and the densities of rgRSV virus NS1 protein were detected by Western blotting using polyclonal antibody with anti–panantigen of RSV virus. A549 cells pre–transfected with siRSV NS1, but not siHPV18 E7, showed a significant reduction in the expres–

sion of viral NS1 proteins (Fig. 1B). To test whether this plasmid is functional and capable of suppressing gene expression, HEK293 cells were cotransfected with pAAV-EGFP, a plasmid expressing green fluorescent protein and pSMWZ-siEGFP (siEGFP), and the percentage of cells expressing EGFP was quantified. The results showed that there was a significantly silencing of EGFP expression (Fig. 1C). In contrast, cells cotransfected with siRSV NS1 in place of siEGFP did not show any reduction in EGFP expression.

[0023] *siRSV NS-1 inhibits the production of rgRSV in human A549 cells in a dose dependent manner.* To test siRSV NS1 candidate, A549 cells were transfected with either siRSV NS1 or siHPV18 E7, then 1 day post-transfection infected with rgRSV at an MOI of 1. At 1 day post-infection, the numbers of rgRSV virus infected cells were quantified by fluorescence microscopy or by flow cytometry. The decrease in the percentage of cells expressing EGFP showed that there was a silencing of rgRSV virus production in a dose-dependent and sequence-specific manner (Fig. 2A, 2B).

[0024] *siRSV NS-1 decreases virus titers and virus replication* To test whether the reduction in the number of infected A549 cells and the amounts of viral proteins expression in-

volved a reduction in rgRSV virus titer, the culture supernatants were examined using an A549 cell-based modified plaque assay. SiRSV NS1 significantly decreased rgRSV virus titer compared to control (Fig. 3A). These results indicate that siRSV NS1 can significantly decrease rgRSV virus production in human A549 cells. To determine whether si-RSV NS1 decreases virus replication, a RT-PCR analysis was conducted. As shown in Fig. 3B, NS1 genes in the antigenome were reduced by siRSV NS1 treatment compared with positive control and non-specific control (siHPV18 E7), and the decreases was showed in dose-dependent fashion, indicating that siRSV NS1 can be used to suppress RSV replication in A549 cells model.

[0025] *Measurement of cell proliferation on A549 cells transfected with plasmid DNA* To assess whether the various amounts of plasmid DNAs used in this study influence the viability of A549 cell, MTT assay was used to detect the proliferation on A549 cells. In this assay, the mitochondrial dehydrogenase enzymes of living cells cleave the tetrazolium ring of the yellow MTT to form purple formazan crystals, which are insoluble in aqueous solutions (14). The crystals were dissolved in acidified isopropanol, and the optical density of resulting purple solution was spectrophotometrically

measured at 570 nm. An increase or decrease in the viable cell number results in a concomitant change in the amount of formazan formed, indicating the degree of viable cells caused by the indicated dose of plasmid DNAs. Results of this study indicated that the treated A549 cells were reduced in cell growth after transfection by different dose of plasmids used in this study, compared with the plasmid DNA-free control group. However there was almost the same metabolic viability among these transfected groups (Fig. 4), which suggesting the inhibitory effects observed in our study were due to nonspecific effects on cellular proliferation induced by plasmid DNA transfection.

[0026] *Si-RSVNS1 treatment augments IFN- α response in RSV-infected cells.* RSV NS1 gene interferes with host cells type-I IFN response. To examine whether hosts cells response to RSV infection is altered in response to si-RSVNS1 treatment, cells were lysed following RSV infection and the proteins were western blotted for various IFN-related response genes, including STATs, STAT1, STAT2 and STAT6 and IRFs, IRF1, IRF3 and IRF7. The results show that the expression of these genes, with the exception of STAT2, are augmented when cells are treated with si-RSVNS1 com-

pared to control (Figure 5).

[0027] DISCUSSION

[0028] Transcriptional mapping studies using UV light showed that RSV genes are transcribed in the order 3' to 5' from a single promoter near the 3' end (15). And the transcription by nonsegmented negative-strand viruses is regarded as a polar model; the promoter-proximal genes are transcribed more frequently than the promoter-distal ones (16). NS genes of RSV locates at the 3' end of the viral genome and do not have counterparts in other Paramyxoviridae genera (1). The transcript of NS1 is most abundantly expressed RNAs in RSV-infected cells. However, only small amounts of NS1 protein can be detected in purified virions (17, 18). The NS1 gene of RSV strain A2 is 552 nt long and encodes a protein of 139 amino acids and about 15KD by Mr (6). The deletion of NS1 gene severely attenuates RSV infection in vivo (9, 10, 19), indicating that decreased NS1 protein expression would be expected to decrease the amount of viral replication. Since the processes of RSV transcription and replication are thought to use the same promoter, NS1 might act at a common early stage such as initiation at the genomic (and antigenomic) promoter (16).

[0029] Studies on bovine RSV showed that NS1 is an antagonist of type I interferon-mediated antiviral state (20, 21, 22). And, NS1 has been also regarded as a potent inhibitor of RSV minigenome transcription and RNA replication (16). Therefore, the exact function of NS1 protein remains unclear so far.

[0030] Previous studies demonstrated that A549 cells respond to RSV infection similarly to primary airway cells in culture (23, 24). In this study, we utilized a DNA vector-based system to deliver RSV NS1 siRNA into A549 cells and estimated its anti-RSV effect in vitro. RNA interference is triggered by dsRNA that is cleaved by an RNase-III-like enzyme, Dicer, into 21–25 nucleotide fragments with characteristic 5' and 3' termini (25). These siRNAs act as guides for a multi-protein complex, including a PAZ/PIWI domain containing the protein Argonaute2, that cleaves the target mRNA (26). These gene-silencing mechanisms are highly specific and can potentially inhibit the gene expression of different viruses (27, 28, 29, 30). The specific silencing of pathogen genes using siRNA is a very attractive approach for the clinical treatment of infectious diseases. Long dsRNAs (of >30 nt in length) activate a dsRNA-dependent protein kinase and 2',

5'-oligoadenylate synthetase in mammalian cells, which leads to a non-specific reduction in levels of mRNAs (31). The endogenous expression of siRNAs from introduced DNA templates is thought to overcome some limitations of exogenous siRNA delivery, in particular its transient effects on silencing specific genes and loss of phenotype (32). The major challenges of developing a safe, effective delivery system are achieving the appropriate vector encoding exogenes, which allow transient siRNA expression in vitro and in vivo, leading to specific viral gene silencing.

[0031] The non-viral approach to gene expression has a number of advantages, including ease of use and preparation, stability and heat resistance. The plasmids do not replicate in mammalian hosts and do not integrate into host genomes, yet they can persist in host cells and express the cloned gene for a period of weeks to months. In this vector system, we incorporated the mouse U6 promoter, which is important for transcription and folding of the suppressor RNA, into a plasmid pCMV-U6 to produce pSMWZ-1, and tested this vector with a siRNA cassette composed of a nucleotide sequence from the NS1 gene of RSV. The results obtained in our cells model showed that the transfection ratio was about 42% for pAAV-EGFP plas-

mid. Although siHPV18 E7 was also showed to inhibit rgRSV production, in some degree, in A549 cells using flow cytometry assay (26.82%), siRSV NS1 showed much stronger in the inhibitory efficiency (89.84% for 2 μ g and 97.39% for 4 μ g, respectively) (Fig.2B). To identify whether NS1 inhibition came from siRSV cassettes specifically, siRSV NS1 was used to cotransfect HEK293 cells with pAAV-EGFP plasmid.

[0032] As shown in Fig.1B and 1C, EGFP protein expression was not reduced by siRSV NS1 treatment. Furthermore, NS1 protein was also analyzed using western blotting assay. As shown in Fig. 2C, NS1 protein expression were silenced significantly by siRSV NS1 compared with siHPV18 E7 control and RSV positive control. To assess whether the various reduction in rgRSV production was related to the cell growth induced by plasmid DNAs, MTT assay was used to detect the proliferation of A549 cells and the result showed that the growth of A549 cells were suppressed to some extent for all the plasmids we used (Fig.4), that might partly result in the decreases of rgRSV production in A549 cells for some unclear reasons. That is in accordance with the result siHPV18 E7 was showed to reduce rgRSV production in A549 cells nonspecifically in this

study (Fig. 2B). Moreover, for the investigation of the event of rgRSV replication in the cells transfected with siRSV NS1, RT-PCR quantification analysis was performed and the results showed that reduction in NS1 RNA was special and in dose-dependent decrease in viral antigenome (Fig. 3).

[0033] Our data supports the suggestion that the promoters for RSV transcription and replication involve overlapping sets of nucleotides at the very 3' end of the genome and its downstream gene start sequence NS1 (33). The results indicate that siRSV NS1 may be used to attenuate RSV infection in human respiratory derived cells and may have therapeutic value.

[0034] The method of delivery of administration of siRSVNS is significant as this will ensure that sufficient siRNA will be present de novo when the incumbent virus infects these cells. This can be achieved either transfecting the nasal epithelium with a nonpathogenic recombinant adenoassociated virus or by a polymeric nanoparticles with a frequent administration to ensure sufficient siRSVNS is expressed de novo to attenuate incumbent viral infection.

[0035] In an example, mice are infected with recombinant adeno-associated virus (rAAV) expressing siRSVNS, i.e. a combi-

nation of siNS1 and siNS2 and then mice will be infected with RSV two–three weeks after AAV infection. Five days after their titer in the lung is examined. In another example, mice are administered with polymeric nanoparticles containing plasmid expressing siRSVNS, i.e. a combination of siNS1 and siNS2 and then mice will be infected with RSV within a week of treatment. Five days after their titer in the lung is examined.

[0036] REFERENCEThe following citations are incorporated herein by reference:1.Collins et al 2001. P.L. Collins, R.M. Chanock and B.R. Murphy, Respiratory syncytial virus. In: D.M. Knipe, P.M. Howley and D.E. Griffin, Editors, (4th ed ed.), Fields Virology Vol. 1, Lippincott–Raven, Philadelphia (2001), pp. 14431485.

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[0040] It is also to be understood that the following claims are intended to cover all of the generic and specific features of the invention herein described, and all statements of the scope of the invention which, as a matter of language, might be said to fall therebetween. Now that the invention has been described,

Claims

- [c1] A method of attenuating respiratory syncytial virus comprising the step of transfecting cells to with DNA vector-based short interference RNAs.

Prophylactic Antiviral and Immunomodulatory Therapy for Prevention of Respiratory Syncytial Virus Infection

Abstract

The method of anti-sense gene therapy using viral and non-viral vectors expressing gene(s), oligonucleotide (s) with intent to down regulate gene expression, a specific RSV genes, NS1 and NS2.

These vectors permit suppression of gene expression by gene-specific siRNA cassettes and consequently respiratory syncytial virus replication. Down regulation of NS1 gene upregulates IFN-related protection provided by the host cells.

Fig. 1A

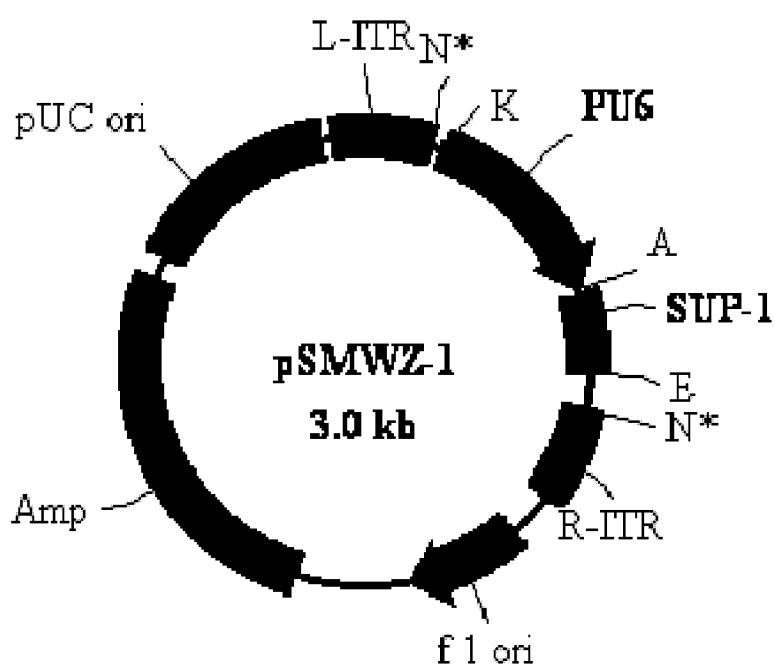


Fig. 1B

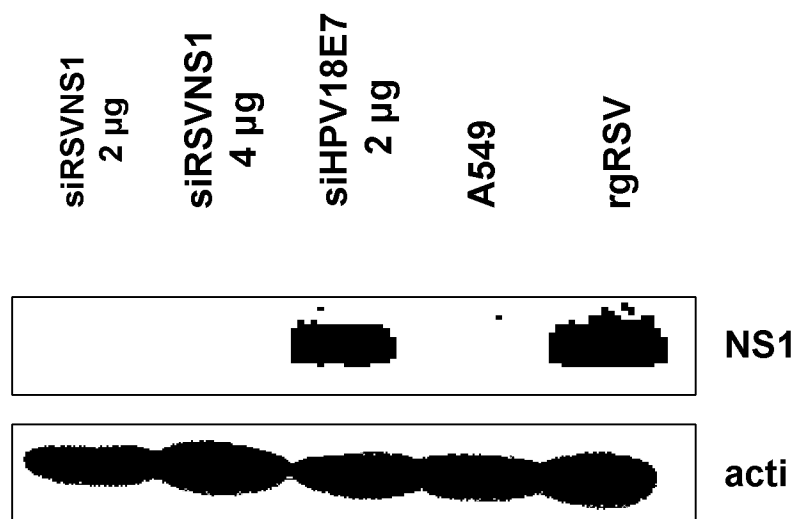


Fig. 1C

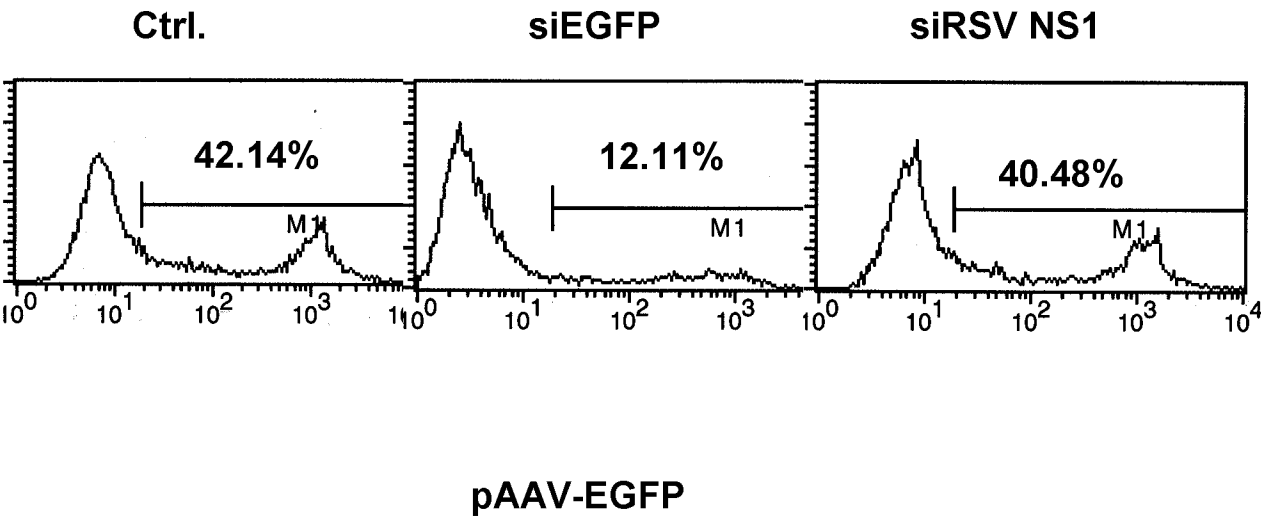


Fig. 2A

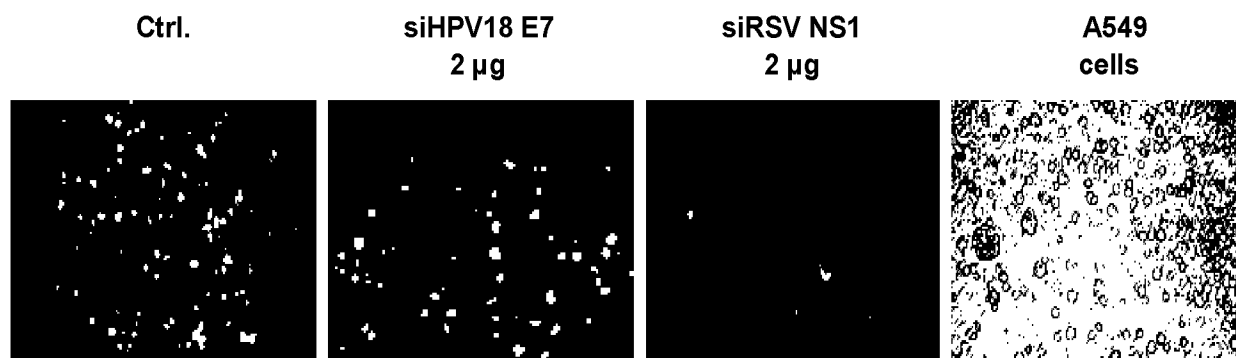


Fig. 2B

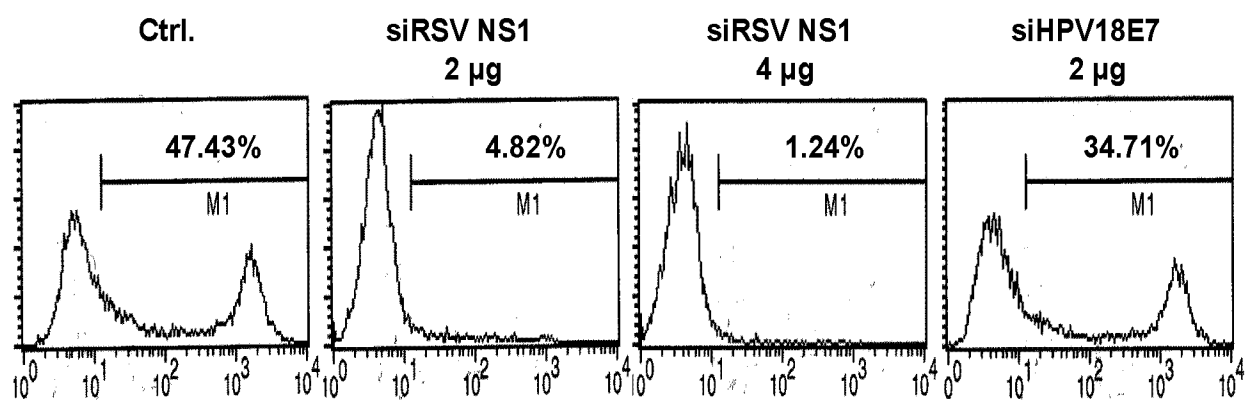


Fig. 3A

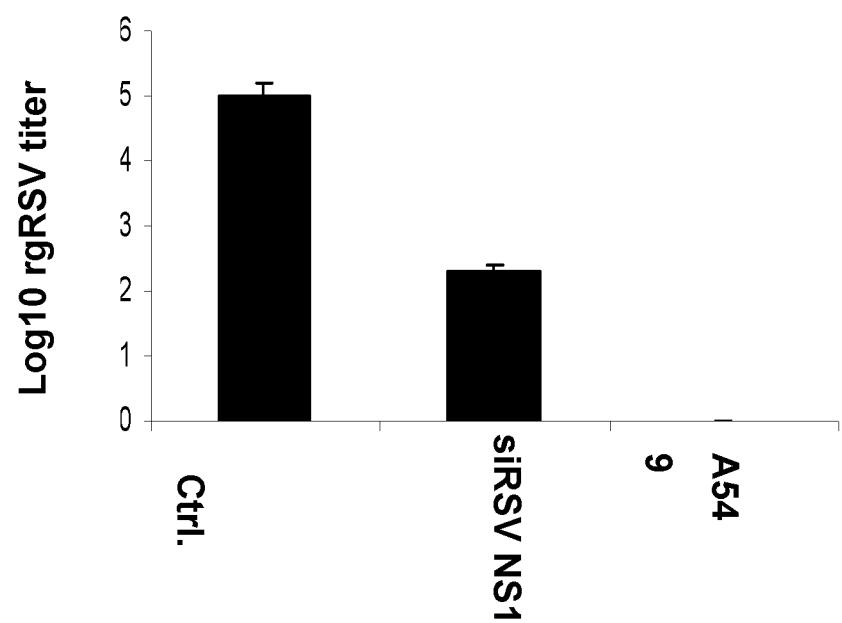


Fig. 3B



Fig. 4

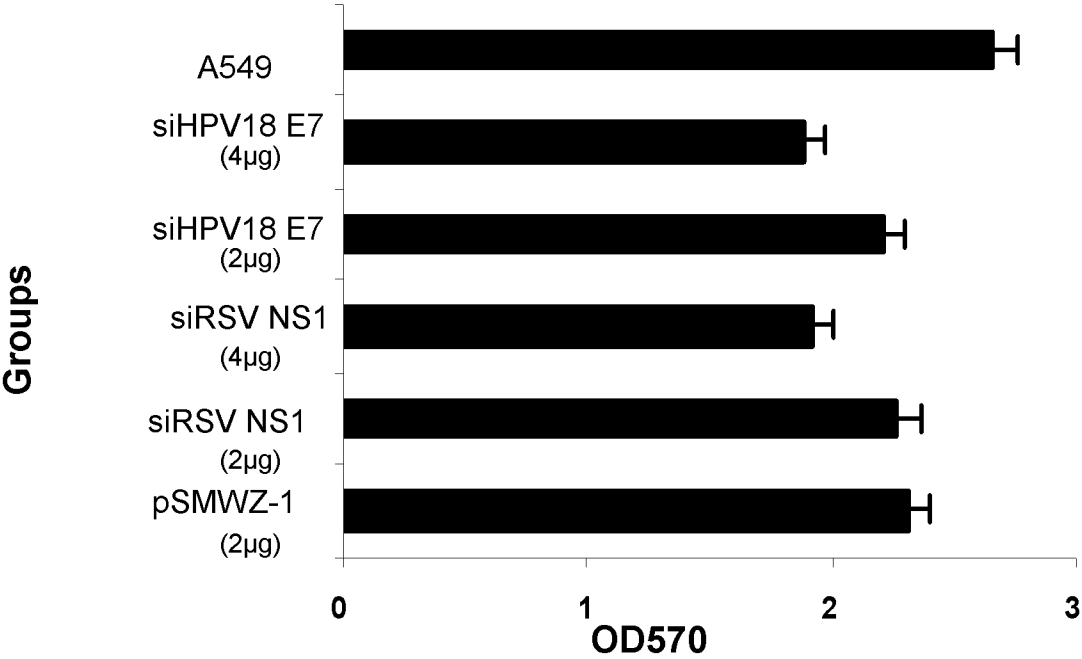


Fig. 5

